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Cell-Specific, Promoter-Dependent Mineralocorticoid Agonist Activity of Spironolactone

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SUMMARY

The agonist activity of the antimineralocorticoid spironolactone was evaluated in various cell lines through the use of transfection experiments. The target promoters were derived from the ΔMTV promoter in which one or several glucocorticoid-responsive elements (GRE) were inserted in tandem. Spironolactone at 100 nM activated by 6-fold the GRE/ΔMTV promoter in the human hepatoma HepG2 cell line and only partially prevented the 10-fold activation of this promoter by 0.1 nM aldosterone. Both effects were completely dependent on the cotransfection of an expression vector for the mineralocorticoid receptor. The half-maximal agonist effect of spironolactone was similar to its half-maximal antagonist effect (~10 nM). For the GRE-2/ΔMTV, GRE-4/ΔMTV, and wild-type MMTV promoters, the activation by aldosterone was much more potent (70-, 100-, and 110-fold, respectively), whereas spironolactone elicited a 10-, 24-, and 25-fold activation, respectively. Thus, the effect of both compounds and the relative efficiency of spironolactone, compared with that of aldosterone, were dependent on the number of GREs present in the regulatory region of the promoter. The agonist effect of spironolactone was cell specific. Indeed, although spironolactone agonist activity was observed in H5 kidney tubule cells, none could be detected at concentrations of ≤1 μM in the CV1 monkey fibroblast cells. In contrast, the antagonist effect was observed in all cells. Furthermore, other antimineralocorticoids, such as RU 26752 and progesterone, also displayed mineralocorticoid receptor-dependent agonist activity in the HepG2 cells. The antiprogesterone RU 486 and the antiandrogen cyproterone acetate were ineffective at ≤1 μM. In conclusion, we show that under certain experimental conditions, several antimineralocorticoids display significant agonist activity in a cell-specific and promoter-dependent manner.

Steroid hormones have essential roles in a wide variety of physiological functions, such as the regulation of sugar and hydromineral homeostasis, reproduction, and the control of cell division and differentiation. They act through intracellular receptors that have been cloned and characterized (1). Antagonists have been developed to counteract the effects of these hormones, particularly in hormone-dependent cancers (e.g., estrogens, androgens), hypertension (e.g., mineralocorticoids), and reproduction (e.g., progestins).

Steroid hormone antagonists have been classified as either pure antagonists or partial agonists depending on their ability to activate the corresponding receptors under certain conditions (2). The latter type includes some of the most studied antagonists, such as RU 486 and tamoxifen. The steroid analog RU 486 is a potent antagonist of progesterone and glucocorticoids (3); it binds with high affinity to either the progesterone receptor or the GR. However, RU 486 acquires agonist activity, particularly when the cAMP signaling pathway is stimulated (4). In the case of antiestrogens, the potency of tamoxifen as an estrogen antagonist depends on the tissue and response examined (5). The ability of tamoxifen to act as a partial estrogen agonist results from its ability to promote ER binding to DNA, thus allowing the domain containing the constitutive transcription activation function of the receptor to activate transcription. The demonstration of partial agonist activity for antisteroids is important because it contributes to the elucidation of their mechanism of action and because of the clinical applications of these compounds.

The spirolactone class of compounds was synthesized during the late 1950s to block aldosterone, the endogenous steroid hormone affecting the homeostasis of sodium, potassium, and hydrogen ions (6). Spirolactones inhibit the effects

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ABBREVIATIONS: GR, glucocorticoid receptor; GRE, glucocorticoid-responsive element(s); ER, estrogen receptor; MR, mineralocorticoid receptor; DMEM, Dulbecco's modified Eagle's medium; CAT, chloramphenicol acetyltransferase; hMR, human mineralocorticoid receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CoA, coenzyme A.

of aldosterone and deoxyaldosterone on electrolyte excretion in rats and induce natriuresis in humans. A specific spirolactone, spironolactone [7α-(acetylthio)-3-oxo-17α-pregn-4-ene,21 carbolactone], has been a predominant form of this class of drugs and has been used clinically for the past 30 years in the treatment of sodium-retaining states and as an antihypertensive agent (7).

Spironolactone inhibits the effects of aldosterone primarily by competing for its binding site on the MR (6). After its binding to the MR, aldosterone triggers the translocation of the receptor into the nucleus and promotes its binding to cognate responsive elements that are similar to those of the glucocorticoid, progesterone, and androgen hormones (8–10). After binding to DNA, the hormone/receptor complex stimulates the transcription of target genes. The precise step in receptor activation that is blunted by spironolactone is still questionable. Several studies have established distinct effects of agonists and antagonists on the conformation of the receptor, its hetero-oligomeric structure, and its subcellular localization (11–14). One way of addressing this question is to define conditions under which spironolactone displays agonist activity. If such conditions were actually found, this would imply that spironolactone can promote the binding of the receptor to its DNA sites. As we show here using transfection assays, this is indeed the case because spironolactone displays significant agonist activity that is dependent both on the type of promoter and on the cell line used.

Materials and Methods

Cell culture. The human hepatoma cell line HepG2 (15) was maintained in DMEM supplemented with 10% fetal calf serum (GIBCO, Grand Island, NY), 100 units/ml penicillin, 100 μl/ml streptomycin (Diamant, Puteaux, France), and 0.5 μg/ml fungizone (Squibb, Princeton, NJ).

CV-1 monkey kidney cells and COS-1 cells were grown in the medium described above. MCF-7 cells were maintained in DMEM without red phenol. The human kidney tubule cells (H5) were isolated as described by Prié *et al*. (16) and grown in a medium composed of DMEM/Ham's F-12 (1:1) supplemented with 5 μg/ml insulin, 5 μg/ml transferin, 2 mM glutamine, 100 UI/ml penicillin, 100 μg/ml streptomycin, 20 mM HEPES, 0.5 μg/ml fungizone, and 2% charcoal-stripped fetal calf serum.

Plasmids. The human GR and MR expression vectors (RSV-GR, RSV-MR) were a generous gift from Dr. R. Evans (San Diego, CA) (17). The plasmid ΔMTV-CAT was derived from the plasmid MMTV-CAT by deletion of the sequence from position −190 to −88 of the mouse mammary tumor virus long terminal repeat. It was a gift from Dr. Evans, and its construction was described by Umesono *et al*. (18). A *Hin*dIII site, created at the deletion site, was used as a cloning site for all of the oligonucleotides used in this study. The double-stranded oligomers (GRE, GRE-2, and GRE-4) have 5′ extensions that are compatible with a *Hin*dIII site. However, the restriction site is lost in the recombinant plasmid. The GRE-4 sequence was obtained by the ligation of two GRE-2 oligonucleotides into the *Hin*dIII site of the ΔMTV-CAT plasmid. The sequence of GRE was strand A, 5′-AGCTGCTCAGCT GGTACA CTC CGTCCT CTACT-3′; and strand B, 5′-AGCTAGTAG AGGACG GAG TGTACC AGCTGAGC-3′. The sequence of GRE-2 was strand A, 5′-AGCTGCTCAGCT GGTACA CTC CGTCCT ATTATC GGTACA CTC CGTCCT ATTATCTACT-3′; and strand B, 5′-AGCTAGTAGATAAT AGGACG GAG TGTACC GATAAT AGGACG GAG TGTACC AGCTGAGC3-′ (GRE half-sites are underlined). The GRE sequence that we used was derived from the promoter of the aspartate aminotransferase gene (19); it had the same efficiency in transcription as a consensus GRE sequence. The luciferase plasmid (SV40-Luc) was purchased from Promega (Madison, WI).

Transfection experiments. Transfection experiments were performed as described by Garlatti *et al*. (20) with some modifications. One day before the transfection, HepG2 cells (10^6 cells/10-cm dish) were seeded onto the usual culture medium containing 10% fetal calf serum. Ten milliliters of fresh medium with 10% charcoal-treated serum was added to the cells 2–3 hr before the transfection. The CAT plasmids (5 μg of DNA), the hMR or human GR expression vectors (1 μg and 10 ng, respectively), and the luciferase expression vector (1 μg) were introduced into the cells by the calcium phosphate coprecipitation technique followed by a glycerol shock. After the glycerol shock, 10 ml of fresh medium containing 5% charcoal-treated serum was added to the cells. Sixteen hours later, serum-free medium was added, and cells were then treated with the various hormones or drugs tested. After an additional 24-hr incubation, cells were homogenized for CAT and luciferase assays.

A similar transfection protocol was used for CV1 cells (6.10^5 cells/10-cm dish) using different amounts of transfected DNA: 10 μg of CAT plasmid, 2 μg of hMR expression vector and 10 μg of luciferase expression vector. In this case, no glycerol shock was performed. Furthermore, during the treatment with the various drugs, serum was not removed from the culture medium because it was essential for the survival of these cells. The H5 cells were transfected using a similar protocol, but they were seeded at 10^6 cells/3-cm dish. COS-1 cells were transiently transfected by a DEAE-dextran procedure as described by Moyer *et al*. (21). The H4IIEC3 line of the Reuber H35 hepatoma were transfected as described by Aggerbeck *et al*. (22). MCF-7 cells (human breast adenocarcinoma) were seeded at 10^6 cells/6-cm dish and transfected by calcium-phosphate method (23).

Luciferase assay. Luciferase activity was used to normalize the transfection efficiency in all culture dishes (24). It was assayed using a kit from Promega according to the manufacturer's instructions. Briefly, the transfected cells were washed twice with 5 ml of calcium- and magnesium-free phosphate-buffered saline and lysed in 500 μl of Reporter Lysis Buffer 1X (Promega) for 15 min. After a 5-min centrifugation, 20 μl of the supernatant was mixed with 100 μl of luciferase assay reagent (Promega) at room temperature. The luciferase activity was measured using a luminometer 30 sec after the addition of the assay reagent.

CAT assay. The CAT activity was determined using the two-phase assay developed by Neumann *et al*. (25). Briefly, 60 μl of cellular extract that had been heated at 65° for 10 min was incubated with 1 mM chloramphenicol, 30 μl of acetyl-CoA, and 0.5 μCi of [^{3}H]acetyl-CoA (NET-290 L, New England Nuclear Research Products, Boston, MA) at 37° for 30 min. The solution was then transferred to a minivial and layered with 4 ml of Econofluor (NEF 969, New England Nuclear Research Products). After vigorous mixing, the two phases were allowed to separate for ≥15 min, and the radioactivity was then counted in a scintillation counter. Under these conditions, the product of the reaction, acetylated chloramphenicol, but not unreacted acetyl-CoA, can diffuse into the Econofluor phase. For these experiments, blanks were obtained by assaying CAT activity in cells that have undergone the same treatment in the absence of a CAT plasmid.

Results

The mineralocorticoid hormone aldosterone activates the MMTV promoter in HepG2 cells when an MR (hMR) is cotransfected into these cells (Fig. 1A). The maximal activation effect was 110-fold and is achieved at a concentration of 0.1 nM (not shown). Various concentrations of spironolactone were added to the cells in the presence or absence of 0.1 nM aldosterone (Fig. 1A). Spironolactone alone elicited a dose-dependent increase in CAT activity with an EC_{50} value of ≈10 nM. Maximal effect (25-fold) was attained at 100 nM. In

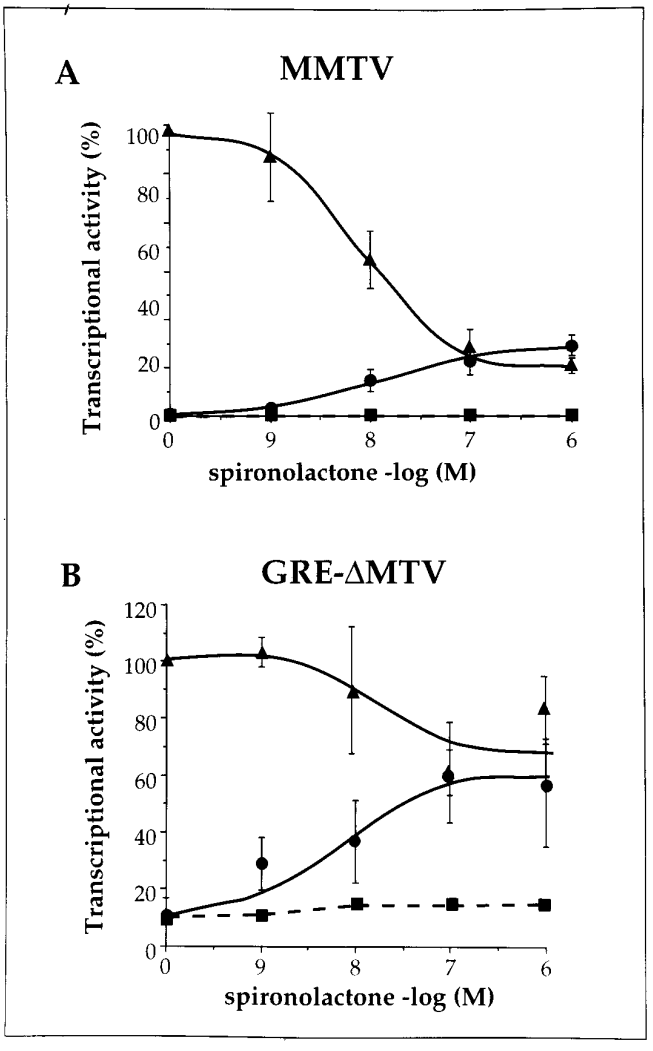

Fig. 1. Agonist effect of spironolactone in HepG2 cells. HepG2 cells were transiently transfected (●, ▲) with or (■) without the hMR expression vector and with the (A) MMTV-CAT or (B) GRE/ΔMTV-CAT plasmids. At 16 hr after the glycerol shock, cells were treated with increasing concentrations of spironolactone (■, ●) alone or (▲) with 0.1 nM aldosterone. Each value is the ratio of the CAT activity to the luciferase activity and is expressed as the percentage of the transcriptional activity in the presence of aldosterone alone. *Points,* mean ± standard error of four separate determinations. In the case of MMTV, 100% activity was 75,600 ± 900 arbitrary units. For the GRE/ΔMTV-CAT plasmid, 100% activity corresponds to 3,900 ± 400 arbitrary units.

the presence of aldosterone, a partial inhibition of the agonist effect was observed and was maximal (80%) at 100 nM (IC_{50} ≈ 20 nM). Thus, the concentration dependence of the agonist and antagonist activities of spironolactone is similar.

To confirm that the agonist effect of spironolactone resulted from its interaction with the MR, HepG2 cells were transfected with the MMTV-CAT reporter plasmid in the presence or absence of an MR expression vector. Fig. 1A shows that spironolactone induced the MMTV promoter in a dose-dependent manner only when an hMR expression vector was cotransfected into these cells. There was no induction in the absence of added hMR.

Spironolactone agonist activity is promoter dependent. One, two, or four GREs were subcloned into the *Hin*dIII site of the ΔMTV promoter (18), yielding the plasmids GRE/, GRE-2/, and GRE-4/ΔMTV-CAT, respectively. The ΔMTV promoter itself was not regulated by aldosterone or spironolactone (data not shown). In the case of the GRE/ΔMTV promoter, aldosterone elicited a 10-fold activation of this promoter (Fig. 1B). Spironolactone displayed both partial agonist and antagonist activities with a concentration dependence similar to that observed for the MMTV promoter. At 100 nM, it activated the GRE/ΔMTV promoter by 6-fold. Interestingly, the agonist effect of spironolactone relative to that of aldosterone on the GRE/ΔMTV promoter was much higher than that on the MMTV promoter (60% versus 20%, respectively; Fig. 1, A and B). This could be related to the fact that aldosterone was less potent in activating the GRE/ΔMTV promoter than in activating the MMTV promoter (10-fold versus 110-fold, respectively; Fig. 2). To further examine this, we compared the efficiencies of spironolactone and aldosterone in activating the ΔMTV promoter containing different arrangements of GREs. In the case of the GRE-2/ΔMTV promoter, aldosterone elicited a 70-fold activation, suggesting that the presence of two GREs in tandem resulted in a synergistic effect (Fig. 2). On the other hand, spironolactone elicited a 10-fold activation, indicating that in this case, the duplication of the GRE yielded, at best, an additive effect. We also tested the GRE-4/ΔMTV promoter, which contains four adjacent GREs. In this case, the effect of aldosterone (≈100-fold activation) was 50% higher than that with the GRE-2/ΔMTV promoter and similar to its effect on the MMTV promoter. The effect of spironolactone (≈24-fold activation) was 2–3-fold higher than that with the GRE-2/ΔMTV promoter (Fig. 2). Thus, the effect of spironolactone relative to that of aldosterone was dependent on the number of GREs present in the regulatory region of the target promoter.

Other GRE-containing promoters were also activated by spironolactone in the HepG2 cells. Spironolactone was 60% as efficient as aldosterone in activating the recombinant GRE/TK-CAT promoter and 13% as efficient as the hormone in activating the natural cAspAT gene promoter (22) (data not shown).

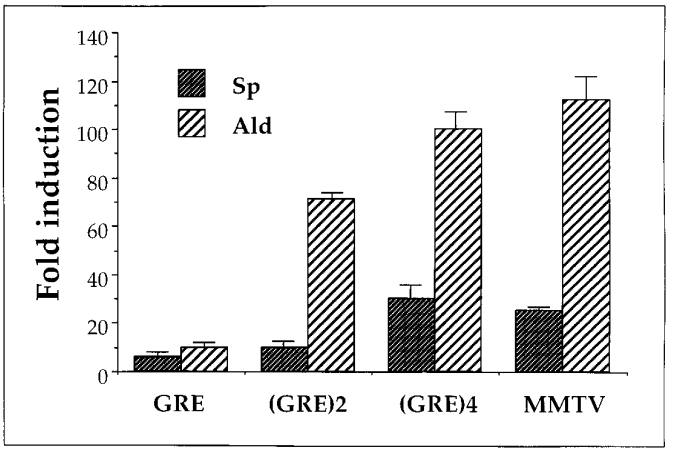

Fig. 2. Promoter-dependent agonist effect of spironolactone. HepG2 cells were transiently transfected with the hMR expression vector and with the GRE/ΔMTV-CAT (*GRE*), GRE-2/ΔMTV-CAT [*(GRE)2*], GRE-4/ΔMTV-CAT [*(GRE)4*], or native MMTV-CAT plasmids. Cells were treated with spironolactone (*Sp*) (0.1 μM) or aldosterone (*Ald*) (0.1 nM). CAT and luciferase activities were determined for each *point*, and the CAT/luciferase ratio was calculated. The results are expressed as the fold induction over basal level. These results are the mean of four independent experiments.

The agonist activity of spironolactone is cell specific. The data presented above were obtained in the human hepatoma cell line HepG2. The activity of spironolactone was also tested in other cell lines. The H5 cell line is derived from the human kidney cortical collecting duct cells, a well known target for mineralocorticoid action. As shown in Fig. 3A, spironolactone displayed significant agonist activity on the GRE-ΔMTV promoter in this cell line too (25% of the aldosterone effect; $EC_{50} \approx 10$ nM).

We also tested the effect of spironolactone in CV1 cells, which originate from monkey kidney fibroblasts and are devoid of functional steroid hormone receptors. In this cell line, aldosterone activated the GRE/ΔMTV promoter in the presence of hMR (Fig. 3B). As expected, spironolactone displayed a dose-dependent inhibition of the aldosterone effect ($IC_{50} \approx 5$ nM) with complete antagonist activity at 100 nM. However, spironolactone exhibited no significant agonist activity, even at high concentrations (≤1 μM). Thus, in CV1 cells, spironolactone displayed pure antagonist properties.

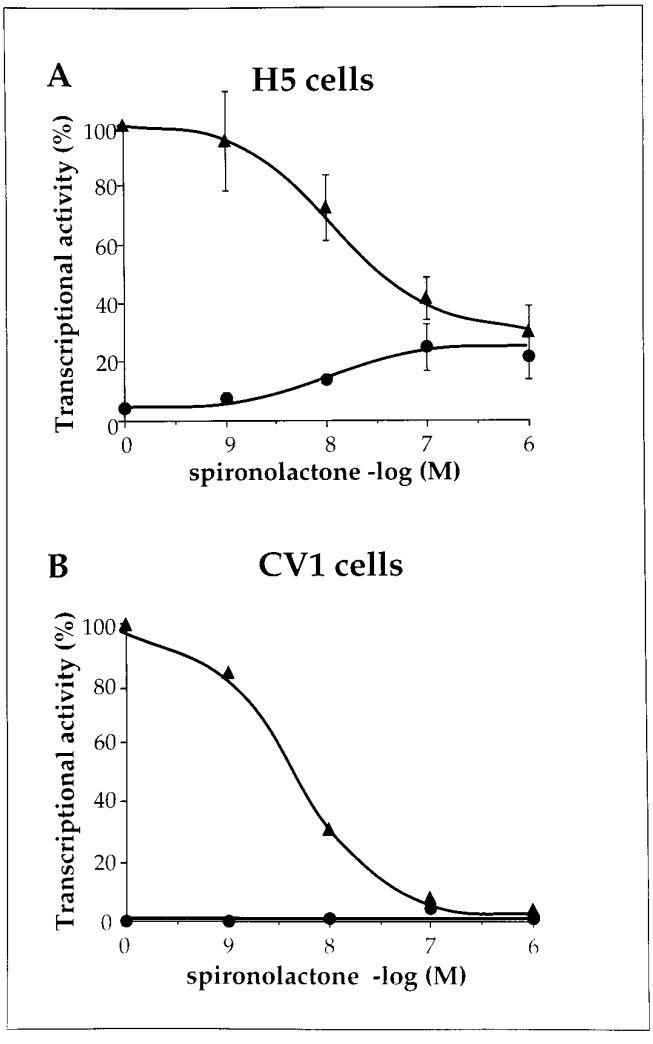

Fig. 3. Cell-specific agonist effect of spironolactone. (A) H5 and (B) CV1 cells were transfected with the hMR expression vector and the GRE/ΔMTV-CAT plasmid. The cells were treated with increasing concentrations of spironolactone in the (●) absence or (▲) presence of aldosterone (0.1 nM). The results were expressed as the percentage of the transcriptional activity in the presence of aldosterone alone. For H5 cells, the aldosterone activity was 1020 ± 130 arbitrary units. In the case of CV1 cells, the aldosterone activity was estimated at 310 arbitrary units.

The effect of spironolactone was examined in additional cell lines. As summarized in Table 1, no agonist effect was detected in the COS-1 cells, another monkey cell line, whereas a small effect was observed in the human mammary tumor cells, MCF7. In addition, a partial agonist effect was found in a rat hepatoma cell line, H4IIEC3. Thus, the presence and the magnitude of the spironolactone agonist effect are dependent on the cell line.

We examined several trivial mechanisms that could account for the cell specificity of the effect of spironolactone. A major difference between the HepG2 and the CV1 cells is that the former, but not the latter, contain functional GR. The possibility that the GR could mediate the effect of spironolactone is unlikely because the cotransfection of an expression vector for the MR is required for its effect (Fig. 1). However, because the formation of GR/MR heterodimers has been reported recently (26), we cannot exclude the possibility that endogenous GR may be necessary for the spironolactone effect.

We verified that the experimental conditions for each cell line did not contribute to the different properties observed for the drug. In particular, during the treatment time, serum was maintained in the culture medium of CV1 cells but not in that of HepG2 cells. The addition of serum to the HepG2 cells during the treatment period did not prevent the agonist activity of spironolactone (not shown).

We also tested for the possibility that an active metabolite of spironolactone could be generated in HepG2 cells but not in CV1 cells. To test this hypothesis, a culture medium containing 1 μM spironolactone was first preincubated with HepG2 cells for various time periods (0, 0.5, 1, 3, 5, 8, and 10 hr) and then transferred onto CV1 cells. These experiments showed that preincubation with HepG2 cells did not trigger the appearance of an agonist activity in CV1 cells (not shown). Therefore, it is unlikely that a diffusible metabolite, generated by HepG2 cells, could account for the agonist activity of spironolactone.

Effect of other antimineralocorticoids. An analog of spironolactone, RU 26752, was tested for agonist activity on

TABLE 1

Effect of spironolactone relative to that of aldosterone in various cell lines

Various cell lines were transfected with the hMR expression vector and with GRE/ΔMTV-CAT or MMTV-CAT reporter plasmids. Cells were incubated in the absence or in the presence of spironolactone (0.1 μM) or aldosterone (0.1 nM). In each case, CAT/luciferase activities have been determined.

Cell line	GRE/ΔMTV			MMTV		
	S	A	S/A	S	A	S/A
HepG2	5.7	9.6	60%	22	112	20%
H5	2.5	10	25%			
MCF-7	5	21.6	21%	6	79	7.5%
H4-II EC3	2.2	11	20%	9	70	13%
CV-1[a]			<5%			<5%
COS-1	1	4.5	<5%	1	5.7	<5%

S, fold induction elicited by spironolactone over the control; A, fold induction triggered by aldosterone over the control; S/A, percentage fold induction elicited by spironolactone relative to that elicited by aldosterone.

[a] For CV1 cells, the basal activity of MMTV and GRE promoters was undetectable, so we could not calculate the fold induction. S/A, ratio of CAT/luciferase activities instead of the ratios of the fold induction elicited by the drugs. For GRE promoter, S = 5 and A = 225. For MMTV promoter, S = 7 and A = 310.

the MMTV promoter. In addition to its expected antagonist activity ($IC_{50} \approx 2$ nM), this compound displayed an agonist activity in HepG2 cells ($EC_{50} \approx 3$ nM) (Fig. 4A) but not in CV1 cells (Fig. 4B). Structurally, RU 26752 differs from spironolactone at position 7, where an *n*-propyl group is present instead of a thioester group. This position is the most likely to undergo metabolic transformation in spironolactone. Despite this structural difference, both compounds are active. Another antimineralocorticoid, progesterone, also displayed agonist activity in HepG2 cells at a concentration of 100 nM (Fig. 5). This effect was dependent on the presence of MR (not shown). Interestingly, RU 486, a well known antiglucocorticoid and antiprogesterone compound, displayed neither antagonist nor agonist activities in HepG2 cells at a concentration of 1 μM. The antiandrogen, cyproterone acetate (1 μM), was also inactive in this experiment (Fig. 5).

Effect of cAMP. cAMP has been shown to trigger the agonist activity of several antisteroids. Thus, we tested the possibility that cAMP could trigger an agonist activity of spironolactone in the CV1 cells. These cells were cotransfected with the GRE/ΔMTV-CAT plasmid and the hMR expression vector and were then treated with various combinations of aldosterone, spironolactone, and 8-bromo-cAMP. As shown in Fig. 6, the addition of cAMP to the CV1 cells did not reveal any agonist activity of spironolactone (Fig. 6). How-

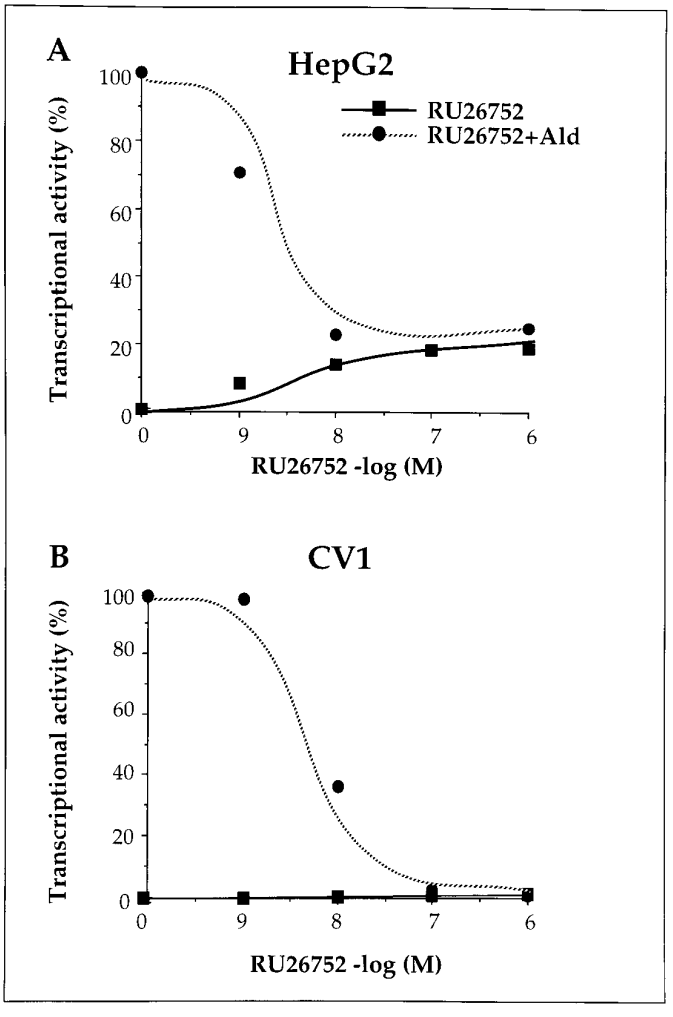

Fig. 4. Effect of the antimineralocorticoid RU 26752. (A) HepG2 or (B) CV1 cells were transfected by the hMR expression vector and the MMTV-CAT plasmid. Cells were treated by increasing concentrations of a spironolactone analog RU 26752 in the (■) absence or (●) presence of aldosterone (*Ald*) (0.1 nM). Results were expressed as the percentage of the transcriptional activity in the presence of aldosterone alone. For HepG2 cells, aldosterone activity was 76,500 arbitrary units, whereas aldosterone activity for CV1 cells was 3,700 arbitrary units. The results are the mean of two independent experiments.

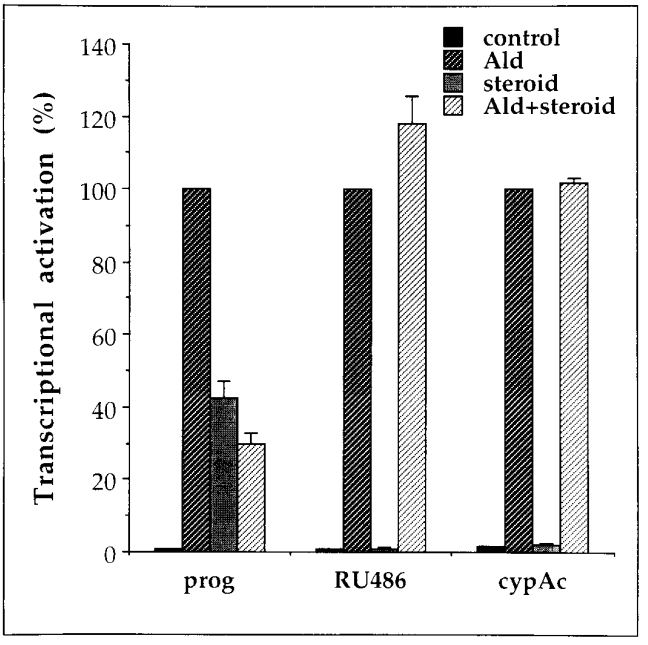

Fig. 5. Effect of various steroids. HepG2 cells were transfected with the hMR expression vector and the MMTV-CAT plasmid. Three sets of experiments are represented; in each, the effects of a different steroid were evaluated: progesterone (*prog*; 100 nM), the antiglucocorticoid RU 486 (1 μM), and the antiandrogen cyproterone acetate (*cypAc*; 1 μM). In each set, transcriptional activity was evaluated in the absence or the presence of aldosterone (*Ald*) alone, the steroid alone, and a combination of aldosterone and the steroid. Results were expressed as the percentage of the transcriptional activity in the presence of aldosterone alone. *Points,* mean of three separate determinations.

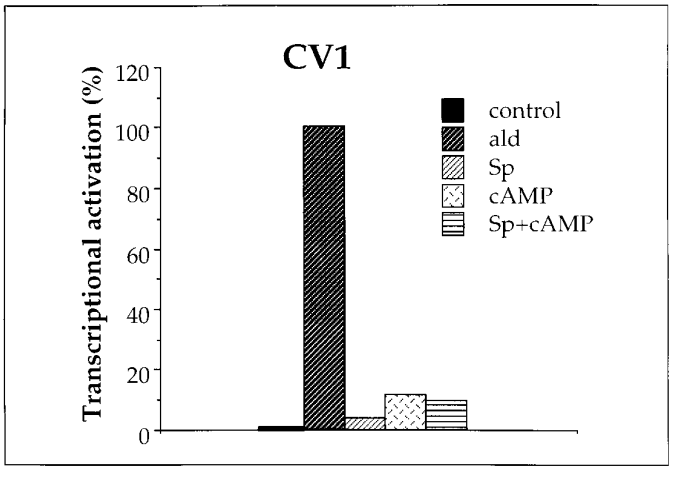

Fig. 6. Effect of cAMP in CV1 cells. CV1 cells were transfected with the hMR expression vector and GRE/ΔMTV plasmid. The cells were then treated with aldosterone (*ald*; 0.1 nM), spironolactone (*Sp*; 0.1 μM), 8-bromo-cAMP (*cAMP*; 0.5 mM), or spironolactone plus 8-bromo-cAMP. Results were expressed as percentage of the transcriptional activity in the presence of aldosterone alone. Aldosterone activity was 301 arbitrary units. *Points,* mean of two independent experiments.

ever, cAMP potentiated the effect of aldosterone in these cells (data not shown).[1]

Discussion

The mechanisms of antisteroid action have been extensively studied in recent years, mostly because of the clinical importance of these compounds. One question that was addressed was whether these drugs display a partial agonist activity under certain conditions. This question is important because of its implications for the efficacy of the drug as well as for understanding the molecular mechanisms of the inhibition of the hormone action. These studies were made possible by the availability of expression vectors for the various steroid receptors and by the use of transfection assays. Using such approaches, it was shown that the antiprogestin and antiglucocorticoid RU 486 (27, 28) and the antiestrogen tamoxifen (29) display agonist activity under certain conditions. In fact, antiestrogens can be grouped into several classes distinguished by their relative agonist activity, reflecting their differential effect on the receptor conformation (30). Much less is known about the properties of the antimineralocorticoid spironolactone; the aim of the current study was to address this.

We showed that spironolactone displays partial agonist activity in several cell lines. This effect is dependent on the presence of MR and is also observed for other antimineralocorticoids. The partial agonist activity of spironolactone leads to an incomplete inhibition of the effect of aldosterone in these cells. In contrast, spironolactone is a very potent antagonist in cells in which no agonist activity could be detected (CV1 cells). Previous studies have failed to establish an agonist activity for spironolactone (6). *In vivo*, this could be explained by the presence of a significant amount of aldosterone in the tissues, in which case only the partial antagonist activity could be seen. Studies on the activation of toad bladder sodium exchange by aldosterone have shown that spironolactone itself acted as an antagonist; however, other related spirolactones displayed some agonist activity (31). There are several differences between those studies and ours: in addition to target and tissue differences, the previous studies were performed during a limited time period (≤), which is much shorter than the period used in the current study. In fact, in HepG2 cells, an increase in CAT activity was barely detectable after 3 hr of treatment and was clearly observed only after 8 hr (not shown).

In a study using the MMTV promoter as a target of mineralocorticoid action in transfection assays, Rupprecht *et al.* (32) reported a potent antimineralocorticoid activity of progesterone and a weak agonist activity of this hormone. In contrast, spironolactone was found to be a pure antagonist. Those studies were performed in the neuroblastoma cells SK-N-MC and support the conclusion that the partial agonist activity of spironolactone that we have observed is cell specific. A similar study was recently conducted by Nordeen *et al.* (33) in the COS-1 cell line. Again, spironolactone alone had no effect on the activity of a promoter derived from the MMTV long terminal repeat promoter in these cells. However, the addition of cAMP triggered partial agonist activity that was weak in the case of spironolactone and strong in the case of another antimineralocorticoid, ZK 30. The discrepancies between the results of the above studies and our study can be explained by differences in the cells and target promoters that were used. Indeed, in our case, the presence of an agonist effect of spironolactone was dependent on the cell type and was not observed in the COS-1 cells.

The mechanism by which spironolactone antagonizes the aldosterone effect is still unclear. It was initially believed that spironolactone competitively inhibits aldosterone binding to the MR and prevents the translocation of the receptor to the nucleus (34). However, Bonvalet *et al.* (35) provided autoradiographic evidence in favor of the presence of the spironolactone/receptor complex in the nucleus. Other studies have confirmed these observations (32, 36) but revealed quantitative differences between agonist- and antagonist-triggered nuclear translocation of the receptor (14). Furthermore, although the effects of aldosterone and spironolactone on the hetero-oligomeric structure of the MR are different, spironolactone was shown to be able to destabilize the interaction between the MR and hsp 90 (12). These two compounds also had a distinct effect on the conformation of the receptor as determined by protease sensitivity (11, 13). Finally, it is also known that a MR/antagonist complex can bind DNA *in vitro* (8). Thus, one model for the antagonist effect of spironolactone predicts that after it binds to the MR, spironolactone, at least partially, triggers the nuclear translocation of the receptor and stimulates its binding to cognate recognition elements forming a complex that was thought to be transcriptionally inactive.

According to the model depicted above, the partial agonist activity of spironolactone can be explained by the ability of the spironolactone/MR complex to interact with DNA *in vivo* and to activate transcription only in certain cells and in a promoter-dependent manner. Several possibilities could account for the fact that the effect of spironolactone is only partial: i) spironolactone could dissociate rapidly from the receptor (12); ii) the nuclear translocation of the receptor/spironolactone complex is less efficient than that of the receptor/aldosterone complex (14); or iii) the conformation of the MR/spironolactone complex is different from that of the MR/aldosterone complex and possibly less efficient in activating transcription. Similar possibilities account for the partial agonist activity of the antiglucocorticoid and antiprogestin RU 486 and of the antiestrogen tamoxifen (5, 28, 37). In the case of the ER, two domains have been shown to be involved in transcriptional activation, TAF I and TAF II (38). TAF II is dependent on the binding of estradiol, but TAF I is constitutive and is predicted to be active if the receptor is bound to DNA even in the absence of estradiol. Because tamoxifen can trigger the binding of the ER to DNA, partial transcriptional activation could occur through TAF I. Interestingly, this effect is cell specific, possibly because of cell-specific cofactors interacting with TAF I (5).

One interesting observation in our study is that the efficiency of spironolactone compared with that of aldosterone depends on the target regulatory region. Indeed, spironolactone was 60% as efficient as aldosterone when a single GRE was present and 15% and 25% as efficient when two or four GREs were present, respectively. This is clearly due to the synergistic effect of GRE duplication on the aldosterone activity (Fig. 2). This effect reaches a plateau when additional

[1] C. Massaad, M. Lombès, M. Aggerbeck, M.-E. Rafestin-Oblin, and R. Barouki. Manuscript in preparation.

GREs are included. In contrast, the effect of spironolactone is additive. This difference could be explained by the ability of aldosterone to recruit different or additional domains of the MR during transcriptional activation compared with spironolactone. Interestingly, a recent study showed that the binding of the ER to two estrogen-responsive elements in tandem was cooperative in the presence of estradiol but not in the presence of tamoxifen (39).

Our observations of a synergistic effect of GRE duplication on the mineralocorticoid induction of transcription apparently contradicts the observations of Rupprecht *et al*. (40). These authors observed a less-than-additive effect when two GREs were linked in tandem. However, there are several differences between the two studies that could account for the discrepancies. Indeed, in some of the cell lines studied, Rupprecht *et al*. used cortisol and not aldosterone to activate the MR. Furthermore, the sequences of the GREs and the surrounding sequences were different. Also, the authors observed that a strong synergism could be detected in their system when an amino-terminal deleted fragment of the MR was used, suggesting a possible contribution of certain MR domains to synergistic induction of transcription.

In conclusion, this study shows that very potent antimineralocorticoids can display partial agonist activity under certain conditions. In this respect, they are similar to other antisteroids that also display such activities. It would be interesting to study other compounds with antimineralocorticoid properties and to classify them as pure antagonist or conditional partial agonists using a cell transfection approach. This could be relevant to the pharmacological properties of these compounds.

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